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MicroRNA-19b Reduces Hepatic Stellate Cell Proliferation by Targeting GRB2 in Hepatic Fibrosis Models In Vivo and In Vitro as Part of the Inhibitory Effect of Estradiol

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ABSTRACT

Estradiol (E2) is a major determinant of gender-based differences in the development of hepatic fibrosis. MicroRNAs (miRNAs) are endogenous 19-25 nucleotide, noncoding, single-stranded RNAs that regulate gene expression by blocking the translation or decreasing the stability of mRNAs and play an important role in liver fibrosis. The mechanisms underlying the regulation of miRNAs by E2 remain largely unknown. In this study, miR-19b levels were higher and were associated with lower GRB2 mRNA and protein levels in female rats more than in male rats. We also showed that miR-19b levels were down-regulated, were associated with the up-regulation of GRB2 mRNA and protein levels in PS (porcine serum-induced hepatic fibrosis) versus NS (normal control) groups and were up-regulated when associated with the down-regulation of GRB2 mRNA and protein levels in PS + E2 versus PS and in aHSC + E2 (estradiol treated aHSC) versus aHSC groups. MiR-19b expression inhibited cell proliferation in aHSCs, and also down-regulated GRB2 protein expression. The overexpression of miR-19b inhibited cell growth and suppressed COL1A1 protein levels by decreasing the levels of GRB2. However, the forced expression of GRB2 partly rescued the effect of miR-19b in the cells, attenuated cell proliferation, and suppressed the GRB2 protein level by up-regulating the levels of GRB2. Taken together, these findings will shed light on the role of miR-19b in regulating aHSC proliferation via the miR-19b/GRB2 axis. This newly identified miR-19b/GRB2 interaction provided novel insights into the suppressive effect of E2 on HSC proliferation and might facilitate the development of therapies targeting hepatic fibrosis. J. Cell. Biochem. 116: 2455–2464, 2015.

KEY WORDS: ESTRADIOL; miR-19b; GRB2; PROLIFERATION

Hepatic fibrosis is characterized by the excessive deposition of extracellular matrix and occurs in a wide range of chronic liver injures as part of the wound-healing process [Wang et al., 2009]. It is well established that the extracellular matrix is mainly derived from hepatic stellate cells (HSCs) in the process of hepatic fibrosis [Priya and Sudhakaran, 2008]. The activation of resident HSCs plays an important role in the pathogenesis of hepatic fibrosis [Priya and Sudhakaran, 2008; Wang et al., 2009]. Interestingly, previous investigations suggested that gender differences existed in the development of hepatic fibrosis [Xu et al., 2002; Shimizu, 2003], which might be due to the inhibitory effect of estradiol (E2) on hepatic fibrosis [Xu et al., 2002; Shimizu and Ito, 2007]. Yasuda et al. [1999] also showed that E2 suppressed hepatic fibrosis by reducing the proliferation of HSCs.

MicroRNAs (miRNAs) are a class of endogenous non-coding, 20–25 nucleotide RNAs that regulate gene expression at the post-transcriptional level by binding the 3′ untranslated region (3′UTR) of target messenger RNAs (mRNAs) [Kim, 2005]. Recently, miRNAs have been shown to be involved in several biological processes such as cell proliferation, differentiation, and apoptosis [Ambros, 2004]. Relevant to this study, miRNAs are HSC regulators that play an important role in hepatic fibrosis [Noetel et al., 2012; Zhang et al., 2012]. The mechanism underlying the regulation of miRNAs by E2 remains largely unknown and requires further study.

According to sequence data from our previous report that were submitted to the NCBI Gene Expression Omnibus under accession number GSE54028 [Shanfei et al., 2014], miR-19b was shown to be significantly reduced in PS (fibrotic) versus NS (normal) samples, and

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Lakner et al. [2012] reported that the expression of miR-19b was down-regulated in a liver fibrosis specimen. As predicted by several in silico methods for target-gene prediction, including TargetScan [Krek et al., 2005], growth factor receptor-bound 2 (GRB2) were identified as a potential target gene of miR-19b. GRB2 is a ubiquitously expressed adaptor protein involved in several tyrosine-kinase dependent signaling pathways [Lowenstein et al., 1992]. As a pivotal signal adaptor protein, GRB2 contributed to cell proliferation by linking other proteins to the membrane after the recruitment of activated EGFR [Baltensperger et al., 1993]. GRB2 also played an important role in fibrosis disease. For example, in lung fibrosis, knocking down the GRB2 protein level could disrupt c-Met receptor downstream signaling, which resulted in diminished HGF-induced ERK-1/2 phosphorylation and the recovery of HGF-inhibited expression of MMP-1, NF-κB, collagen, and CTGF [Bogatkevich et al., 2007]. GRB2 action was essential for cardiac fibrosis in response to regulate fibrosis and cardiomyocyte growth [Zhang et al., 2003].

Herein, we investigated the expression of miR-19b and GRB2 in rat liver stellate cells (aHSC) and liver tissues specimens, which included porcine serum-induced hepatic fibrosis (PS), control (NS), E2-treated (PS + E2), estradiol treated aHSC (aHSC + E2) and vehicle control (aHSC) groups. Moreover, our study confirmed that miR-19b provided a novel mechanism for regulating HSC proliferation through the targeting of GRB2.

MATERIALS AND METHODS

ANIMAL MODELS

Male and female Sprague–Dawley rats were acquired from the Department of Laboratory Animal Science (Xiangya Medical College, Central South University, Changsha, Hunan, China). Male rats were used for the PS-induced liver fibrosis model. All rats were divided randomly into three groups: (1) normal control (NS); (2) liver fibrosis (PS); and (3) E2-treated (PS + E2). NS animals were treated intraperitoneally with 0.5 mL of saline. Liver fibrosis was generated by treating rats (120∼150 g) for 12 weeks with porcine serum (PS) by an intraperitoneal injection of 0.5 mL twice weekly as previously described [Osuna-Martínez et al., 2011a]. E2-treated rats also received the same volume of PS at the same time intervals for 8 weeks, and then the rats were administered estradiol (1 mg/kg; Sigma–Aldrich Corporation, St Louis, Missouri, USA) intraperitoneally twice weekly [Zhang et al., 2012] from the 9th week. After 12 weeks, all rats were sacrificed, and liver tissues were harvested for further analysis. Liver tissues were used for Masson′s trichrome staining by fixation with 10% formalin. All experimental procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences, and approved by the Medicine Animal Care Committee of Xiangya Medical College, Central South University, Changsha, Hunan, China.

HISTOLOGICAL EXAMINATION

Liver tissues were fixed in 10% solutions of formaldehyde in NaCl/Pi buffer (pH 7.4), dehydrated in alcohol and embedded in paraffin. Slides containing four-micrometer sections were prepared and stained with Masson trichromic (MT) stain. Fibrosis percentage were evaluated according to criteria reported elsewhere [Osuna-Martínez et al., 2011b]. The degree of perihepatocellular and luminal fibrosis in each sample were calculated by the following formula* [Osuna-Martínez et al., 2011b]. Histopathologic examination was independently assessed by two board-certified pathologists who were blind to the study:

$$\%\,\text{Fibrosis} = \frac{\text{Fibrosis area}}{(\text{parenchymal area} - \text{luminal area})} \times 100$$

*Fibrosis formula

CELL CULTURE

All cells were cultured in Waymouth MB 752/1 medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco) at 37°C with 5% CO_2. Cells were plated at a density of 2×10^5 cells in 1 mL of culture medium onto uncoated plastic culture dishes. The rat liver stellate cell line (HSC-T6) was originally derived from male Sprague-Dawley rats and became immortalized by transfecting SV40 into rat HSCs as previously described [Vogel et al., 2000]. Sub-confluent aHSCs (obtained from Medical Research Center and Clinical Laboratory, Xiangya Hospital, Changsha, Hunan, China) were cultured in medium containing vehicle controls (aHSC) and 100 nM estradiol (aHSC + E2) (Sigma–Aldrich Corporation, St. Louis, MO, USA) for 48 h [Zhang et al., 2012].

CELL TRANSFECTION

aHSCs were transfected with either miRNA mimics, miRNA inhibitor (GeneCopoeia, USA) or a GRB2 ORF clone (GeneCopoeia, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a final concentration of 50 nM, according to the manufacturer′s instructions. The recommended controls for miRNA mimics and inhibitor (GeneCopoeia, USA) were used in experiments. Changes in gene expression were detected 48 h after transfection.

QUANTITATIVE REAL-TIME PCR (qRT-PCR) ANALYSIS

Total RNA was extracted with TRIzol® reagent (Invitrogen, USA) according to the manufacturer′s instructions. MiR-19b first-strand cDNA was synthesized from 2 μg of total RNA with an All-in-one™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China) using the supplied poly-A primer. RT-PCR was performed in a 20 μL reaction mix including 2 μLof 5 × diluted reverse transcription product, 2 μL of miProfile™ miRNA qPCR primer, 2 μL of miRNA-specific primer, 10 μL of SYBR 2x All-in-one qPCR Mix, 0.4 μL of 50x ROX Reference dye, and 3.6 μL of double distilled water. The cycling conditions for amplification on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. The data were normalized against U6 small nuclear RNA (snRNA).

GRB2 cDNA was synthesized with a RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Canada) in a total volume of 20 μL. GRB2 expression was analyzed with THUNDERBIRD SYBR qPCR Mix (ToYoBo, Japan). The following primer sequences were used: for GRB2, 5′-CCAGGCACTCTTTGACTTTG-3′ (forward) and 5′-CGGTCTCAACTTTTCACTTTC-3′ (reverse); for β-actin,

5′-CTTCCGTGTTCCTCCCC-3′ (forward) and 5′-GCATCAAAGGTG-GAAGAAT-3′ (reverse). qRT-PCR was performed on an Applied Biosystems 7500 RT-PCR System. The cycling conditions for amplification were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, and 55°C for 30 s, and 72°C for 10 s. The data were normalized against β-actin. Each sample was analyzed in triplicate, and the fluorescence signal was measured at each extension step. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method, where the control-normalized CT (ΔCT) value was calculated by subtracting the CT of a control gene (U6 snRNA for miR-19b and β-actin for GRB2) from the CT of the gene of interest.

WESTERN BLOT ANALYSIS

Immunoblotting was performed to detect the expression of GRB2 and COL1A1 in rat liver tissues and aHSCs. Cultured or transfected cells were lysed in RIPA buffer with 1% PMSF. Protein was loaded onto an SDS-PAGE minigel and transferred onto PVDF membrane. After probing with 1:1000 diluted rabbit polyclonal GRB2 and COL1A1 antibody (Abcam, MA, USA) at 4°C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody (1:5000). Signals were visualized using ECL substrates (Millipore, MA, USA). β-actin was used as an endogenous protein for normalization.

LUCIFERASE REPORTER ASSAY

aHSCs were seeded into a 24-well plate. After culturing overnight, the cells were co-transfected with the wild-type 3′UTR reporter plasmid and miR-19b mimics/miR-19b inhibitor and pRL-TK plasmids or were transfected with a mutated GRB2 3′UTR reporter plasmid and miR-19b mimic/miR-19b inhibitor and pRL-TK plasmids. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

CELL PROLIFERATION ASSAY

The cell proliferation assay was carried out using a Cell Counting Kit 8 (CCK8, Beyotime, China). Briefly, cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates and were transfected with 50 nM miR-19b or the GRB2 ORF plasmid. Cell proliferation was assessed after 48 h using CCK8 according to the manufacturer's protocol.

CELL CYCLE ASSAY

Cell cycle analysis was determined using a BD Life Science Research (LSR) FlowCytometer (BD Biosciences). Briefly, aHSCs were cultured at 1×10^6 cells per well in 6-well plates and were transfected with 50 nM miR-19b or GRB2 ORF plasmid for 48 h. Cells were then harvested and fixed in 70% ice-cold ethanol for 24 h, followed by propidium iodide (PI) staining. Different phases of the cell cycle were analyzed using a FACS Calibur™ Instrument.

STATISTICAL ANALYSIS

All data are expressed as the mean and standard deviation from at least three independent experiments. The differences between two or multiple groups were evaluated by *t*-test or one-way analysis of variance (ANOVA). All *P*-values were obtained with the SPSS 17.0 software package (SPSS, Chicago, IL, USA), and a $P < 0.05$ was considered to be statistically significant.

RESULTS

EFFECT OF E2 ON miR-19b EXPRESSION IN LIVER FIBROSIS MODELS IN VIVO AND IN VITRO

The degree of liver fibrosis was determined by Masson's staining (Fig. 1A). Histopathological and morphometric analysis showed that in liver fibrotic tissue sections stained with Masson, PS caused extensive collagen deposition and the formation of regenerative nodules and fibrotic septa between PS groups when compared with sections from livers in the control rats. In contrast, the E2-treated group showed a striking reduction of collagen fibers when compared with the PS group (Fig. 1A and B). Further more, the protein expression of COL1A1 in the PS group was significantly up-regulated versus the NS group and down-regulated in the E2 + PS group (Fig. 1C and D).

To investigate whether the expression of miR-19b was associated with the anti-fibrotic effect of estradiol in liver fibrotic tissues and aHSCs, we first used qRT-PCR analysis to reveal that miR-19b levels were higher in female rats than in male rats. This result indicated that E2 might increase the expression of miR-19b (Fig. 2A). Then, we used qRT-PCR analysis to show that miR-19b was down-regulated in the PS versus the NS group and up-regulated in the PS + E2 versus the PS group (Fig. 2B). Moreover, we investigated the involvement of miR-19b in the E2-treated aHSC group, and showed that E2 increased the expression of miR-19b in the aHSC + E2 versus aHSC groups by qRT-PCR (Fig. 2C). These results indicated that estradiol increases miR-19b expression in liver fibrosis models both in vivo and in vitro.

EFFECT OF E2 ON GRB2 EXPRESSION IN LIVER FIBROSIS MODELS IN VIVO AND IN VITRO

As predicted by several in silico methods for target-gene prediction, including TargetScan [Krek et al., 2005], Growth factor receptor-bound 2 (GRB2) was identified as a potential target gene for miR-19b. To study the effect of E2 on GRB2 expression in liver fibrosis, we investigated the expression of GRB2 in male rat liver specimens, female rat liver specimens, a PS group, a control NS group, a PS + E2 group, aHSCs and E2-treated aHSCs.

First, we used qRT-PCR and western blot analysis to reveal that GRB2 mRNA and protein levels were lower in female rats than in male rats. This result indicated that E2 might decrease the expression of GRB2 (Fig. 3A–C). In addition, western blot and qRT-PCR results further showed that GRB2 levels were up-regulated in the PS versus NS groups and down-regulated in the PS + E2 versus PS groups (Fig. 3D–F). Moreover, we investigated the involvement of GRB2 in the E2-treated aHSC group, and showed that E2 caused decreased expression of GRB2 in the aHSC + E2 versus aHSC groups by qRT-PCR and western blot analysis (Fig. 3G–I). These results indicated that estradiol decreases GRB2 expression in liver fibrosis models in vivo and in vitro and that the expression of GRB2 was negatively related with the expression of miR-19b.

miR-19b INHIBITED THE EXPRESSION OF GRB2 VIA TARGETING THE 3′UTR OF GRB2 IN HSCs

To further study the relationship of miR-19b and GRB2 in aHSCs, we transfected aHSCs with mimics NC and miR-19b mimics. Quantitative RT-PCR showed that 48 h after transfection, the expression of

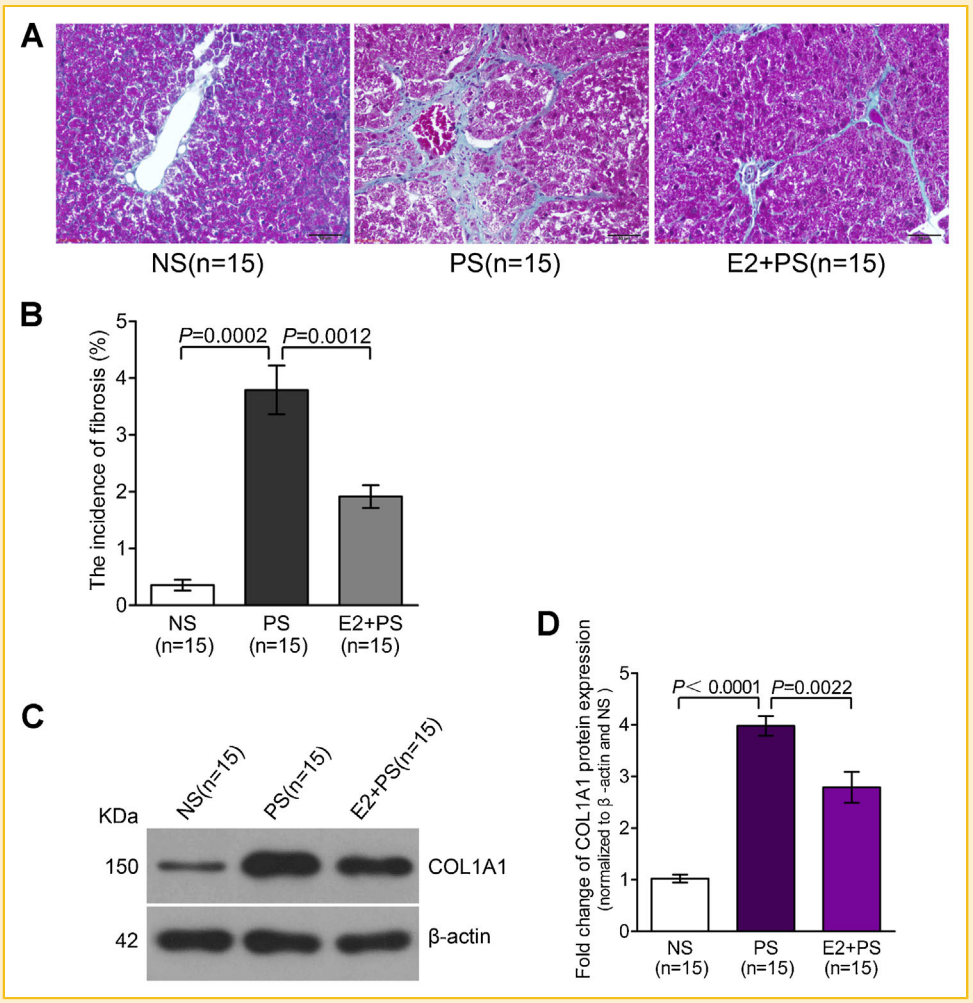

Fig. 1. Effect of E2 on liver histopathology and incidence of fibrosis. (A) Masson's staining of control (NS), porcine serum-induced (PS) and estradiol (E2)-treated (PS + E2) liver tissue. (B) The percentage of incidence of fibrosis was determined by the assay described in the Methods. The data are presented as the mean ± SEM from 15 replicates in each group. (C) COL1A1 protein levels were detected by western blot analysis. COL1A1 protein levels were up-regulated in PS versus NS groups, and down-regulated in PS + E2 versus PS. (D) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to COL1A1 were compared with those corresponding to β-actin. The data are presented as the mean ± SEM from 15 replicates in each group.

miR-19b was up-regulated compared with mimics NC transfected cells (Fig. 4A). While we transfected aHSCs with an inhibitor NC and a miR-19b inhibitor, the miR-19b inhibitor down-regulated the miR-19b level (Fig. 4B). Moreover, western blot results showed that the enhanced levels of miR-19b in aHSC cells suppressed GRB2 protein expression compared with cells transfected with mimics NC, and that receding levels of miR-19b induced the protein level of GRB2 in aHSCs (Fig. 4C, D).

Additionally, to determine whether the 3′UTR of GRB2 mRNA is a functional target of miR-19b in aHSCs, we cloned the 3′UTR of GRB2 downstream of a luciferase reporter gene (wt-GRB2). We also generated a similar construct using a binding site mutant version of GRB2 (mut-GRB2) (Fig. 4E). Then, the wt-GRB2 plasmid and miR-19b mimics or miR-19b inhibitor were co-transfected into aHSCs. The luciferase activity was reduced upon transfection with miR-19b mimics and was induced following transfection with a miR-19b inhibitor (Fig. 4F). Moreover, the miR-19b-mediated repression of luciferase activity was abolished by mutating the putative binding site (Fig. 4F).

miR-19b INHIBITS THE PROLIFERATION OF aHSC THROUGH TARGETING GRB2

To validate whether miR-19b regulated aHSCs proliferation and cell cycle progression by targeting GRB2, we transfected a GRB2 ORF clone into aHSCs to up-regulate the protein level of GRB2 (Fig. 5A and B). We then performed CCK8 and cell cycle assays by co-transfecting miR-19b mimics and the GRB2 ORF clone into aHSCs. CCK8 results showed that the forced expression of GRB2 restored the significant inhibition of cell proliferation by miR-19b (Fig. 5C). FACS results showed that the percentage of cells at G1 phase was increased, and that the percentage of cells at S phase was decreased in response to treatment with miR-19b, and this effect was reversed by transfection

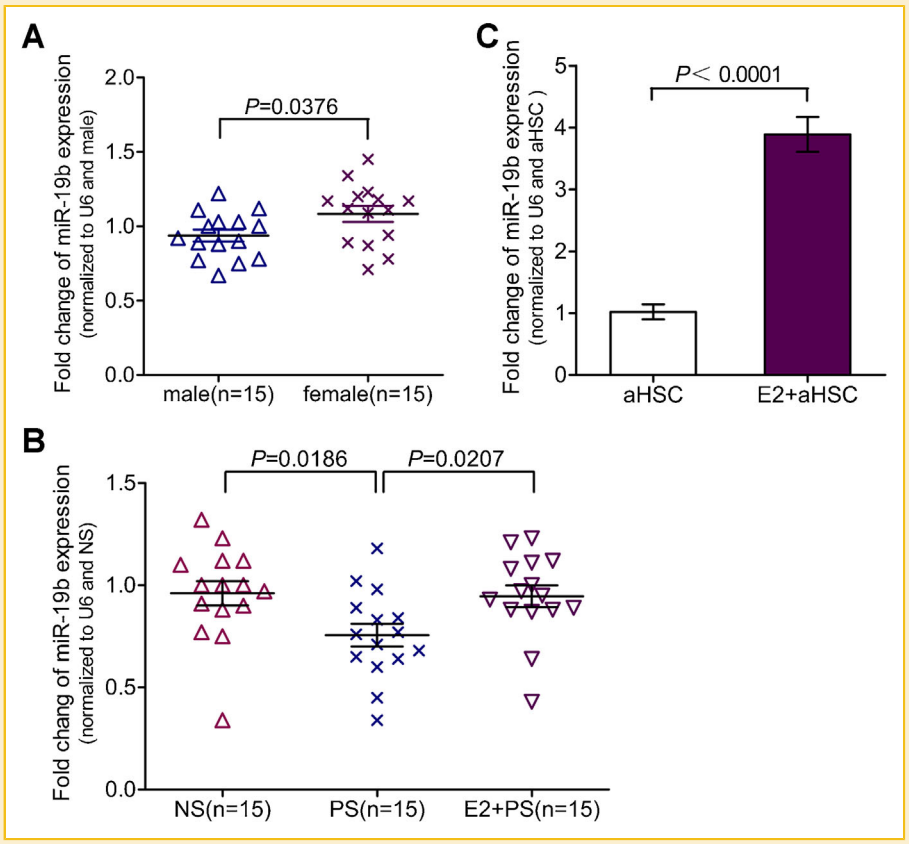

Fig. 2. Effect of E2 on miR-19b expression in liver fibrosis models in vivo and in vitro. MiR-19b expression was analyzed by real-time PCR. (A) miR-19b levels were higher in female rat livers than in male rat livers. (B) miR-19b was down-regulated in the PS versus NS groups and up-regulated in the PS + E2 versus PS groups. The data are presented as the mean ± SEM from 15 replicates in each group. (C) E2 increased expression of miR-19b in aHSC + E2 versus aHSC groups. The data are presented as the mean ± SD from three replicates in each group.

with the GRB2 ORF clone (Fig. 5D, E). Taken together, these results indicated that miR-19b could inhibit the proliferation of aHSCs by targeting GRB2.

miR-19b DOWN-REGULATED THE EXPRESSION OF COL1A1 THROUGH TARGETING GRB2

Activated hepatic stellate cells (HSCs) play a pivotal role in the progression of liver fibrosis and are known to be primarily induced by the abnormal expression of the TGF-beta signaling pathway [Friedman, 2008], which strongly induces the expression of fibrosis-related genes, including COL1A1 [Murakami et al., 2011]. We questioned whether miR-19b could decrease COL1A1 levels by targeting GRB2. As shown in Fig. 6A–C, the forced expression of GRB2 could restore the GRB2 and COL1A1 protein expression inhibited by miR-19b. These results indicated that miR-19b down-regulated the expression of COL1A1 by targeting GRB2.

DISCUSSION

The progression of liver fibrosis is more common in men than in women, and this intrinsic gender difference may be attributed to E2 [Bissell, 1999; Martino et al., 2004]. It has also been shown that E2 treatment reduces HSC proliferation and attenuates hepatic fibrosis [Shimizu et al., 1999; Yasuda et al., 1999]. To date, the molecular mechanism underlying the suppression of hepatic fibrosis by E2 remains unclear. Zhang et al. [2012] showed that E2 inhibited hepatic fibrosis through the induction of hepatic miR-29. However, the role of miRNA in the relationship between E2 and hepatic fibrosis is largely unexplored.

Here, we showed that the expression of miR-19b was down-regulated in PS versus NS groups and up-regulated in PS + E2 versus PS and aHSC + E2 versus aHSC groups. Additionally, real-time PCR and western blot results showed that the expression of GRB2 was up-regulated in PS versus NS groups and down-regulated in PS + E2 versus PS and aHSC + E2 versus aHSC groups. These results suggested that E2 could induce miR-19b expression, which is consistent with microarray and real-time PCR results published by Castellano et al. [2009]. The report also showed that the E2-mediated up-regulation of miR-19b subsequently down-regulated ERα, which transcriptionally modulated several genes implicated in cell proliferation, such as AIB1, c-myc, and cyclin D1 [Rosenfeld et al., 2006]. As a potential target gene for miR-19b, GRB2 was down-regulated by E2 in liver fibrosis, and its protein level was negatively correlated with the miR-19b expression level in vivo and in vitro.

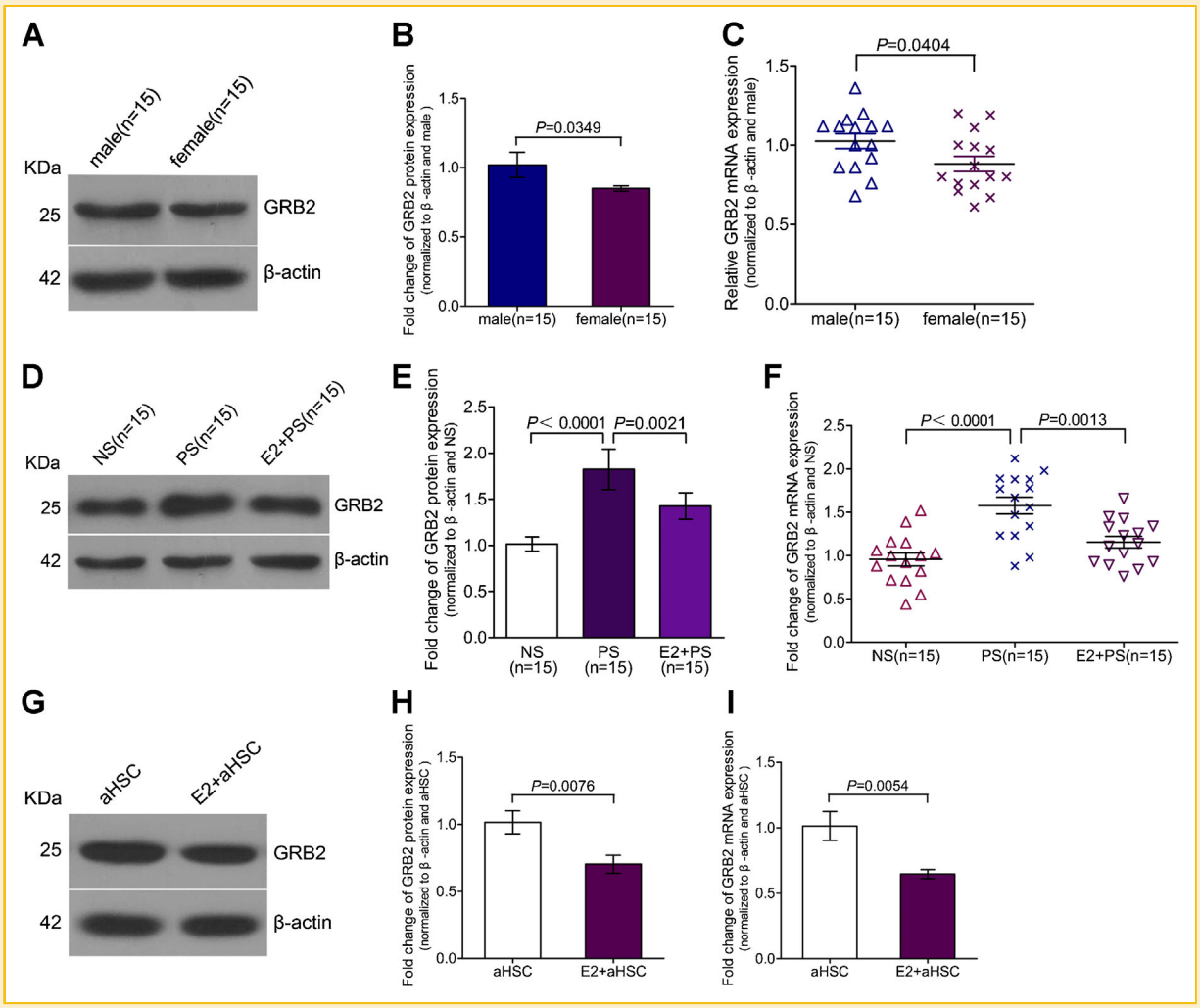

Fig. 3. Effect of E2 on GRB2 expression in liver fibrosis models in vivo and in vitro. GRB2 expression was analyzed by real-time PCR and western blot. (A) GRB2 protein levels were lower in female rat livers than in male rat livers. (B) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared with those corresponding to **β**-actin. The data are presented as the mean ± SEM from 15 replicates in each group. (C) GRB2 mRNA levels was lower in female rat livers than in male rat livers. (D) GRB2 protein levels were up-regulated in the PS versus NS groups and down-regulated in the PS + E2 versus PS groups. (E) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared with those corresponding to **β**-actin. The data are presented as the mean ± SEM from 15 replicates in each group. (F) GRB2 mRNA levels were up-regulated in the PS versus NS groups and down-regulated in the PS + E2 versus PS groups. (G) The protein expression of GRB2 was down-regulated in aHSC + E2 versus aHSC groups. (H) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared with those corresponding to **β**-actin. (I) The mRNA expression of GRB2 was down-regulated in the aHSC + E2 group versus the aHSC group. The data are presented as the mean ± SD from three replicates in each group.

To determine whether miR-19b directly targeted the 3′UTR of GRB2 and whether miR-19b regulated the proliferation of aHSCs via GRB2, we analyzed luciferase activity using a reporter vector containing the two alleles. The luciferase vector with a mutant-type allele showed significantly less activity than the wild-type vector, as shown in Fig. 3. The over-expression of miR-19b decreased the luciferase activity, and the inhibition of miR-19b increased the luciferase activity, suggesting that miR-19b directly repressed GRB2 expression. Transfection with the miR-19b mimics or inhibitors significantly decreased or increased GRB2 protein levels in a precursor miRNA concentration-dependent manner (Fig. 3). It was suggested that miR-19b suppressed the expression of the endogenous GRB2 protein by controlling the stability of GRB2 mRNA transcripts. Taking these observations into consideration, miR-19b acted as a repressive regulator of GRB2.

MiRNAs have emerged as key regulatory molecules in chronic liver disease, including hepatic fibrosis [Chen, 2009; Dolganiuc et al., 2009; Jin et al., 2009]. Array profiling studies reported differential miR expression in normal versus fibrotic liver tissue in a variety of rodent injury models, including BDL (Bile duct ligation) and carbon tetrachloride (CCl4) [Roderburg et al., 2011]. miR-150, miR-187, miR-194, and miR-207 were significantly down-regulated in HSCs isolated from BDL (Bile duct ligation) animals compared with sham controls, whereas let-seven family members were significantly upregulated [Roderburg et al., 2011]. The overexpression of miR-150 and miR-194 in human HSCs (LX-2) resulted in the inhibition of

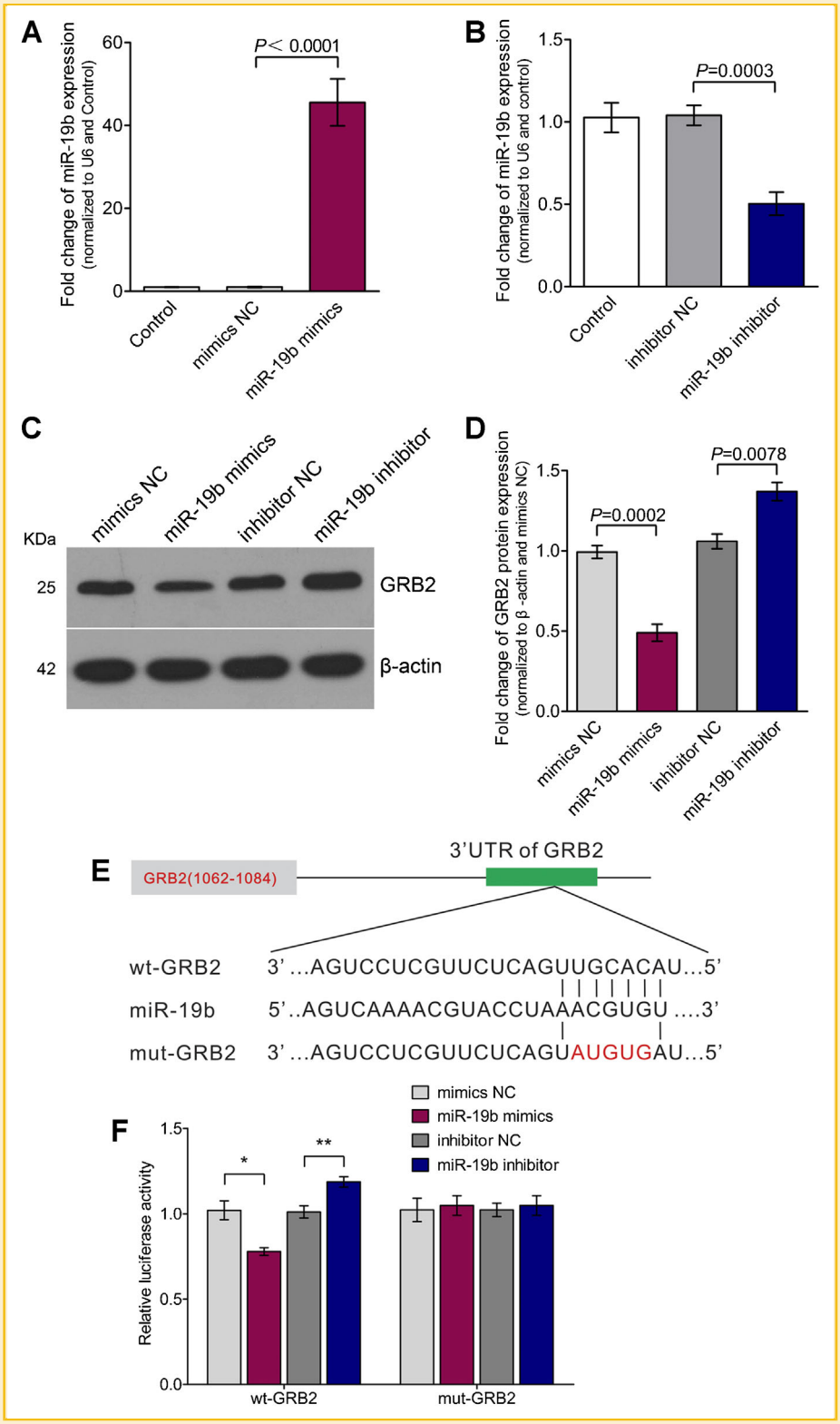

Fig. 4. miR-19b directly targeted GRB2 by binding to its 3′UTR. (A) Up-regulated expression of miR-19b was tested by Quantitative RT-PCR in aHSCs transfected with miR-19b mimics and were comparison with aHSCs transfected with mimics NC. (B) Down-regulated expression of miR-19b was tested by Quantitative RT-PCR in aHSCs transfected with a miR-19b inhibitor and were comparison with aHSCs transfected with an inhibitor NC. (C) GRB2 protein levels were detected by western blot analysis. The expression of GRB2 was decreased by a miR-19b mimics and was increased by miR-19b inhibitor when compared with aHSCs expressing mimics NC and an inhibitor NC. (D) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared to those corresponding to β-actin. (E) The predicted miR-19b binding site within the GRB2 3′UTR and its mutated version by site mutagenesis are shown. (F) Relative luciferase activities of GRB2 wild-type (WT) and mutant (Mut) 3′-prime UTR regions were obtained by co-transfection of a miR-19b mimic or a miR-19b inhibitor and the psiCHECK-2 plasmid and were calculated as the ratio of firefly/renilla activities in the cells and normalized to those of the control. The results are presented as the mean ± SD from three independent experiments with each experiment in triplicate. Double asterisks indicate significant difference when compared to the control. ($^{*}P < 0.01$, $^{**}P < 0.01$). The figure is representative of three experiments with similar results. Values are presented as the mean ± SD.

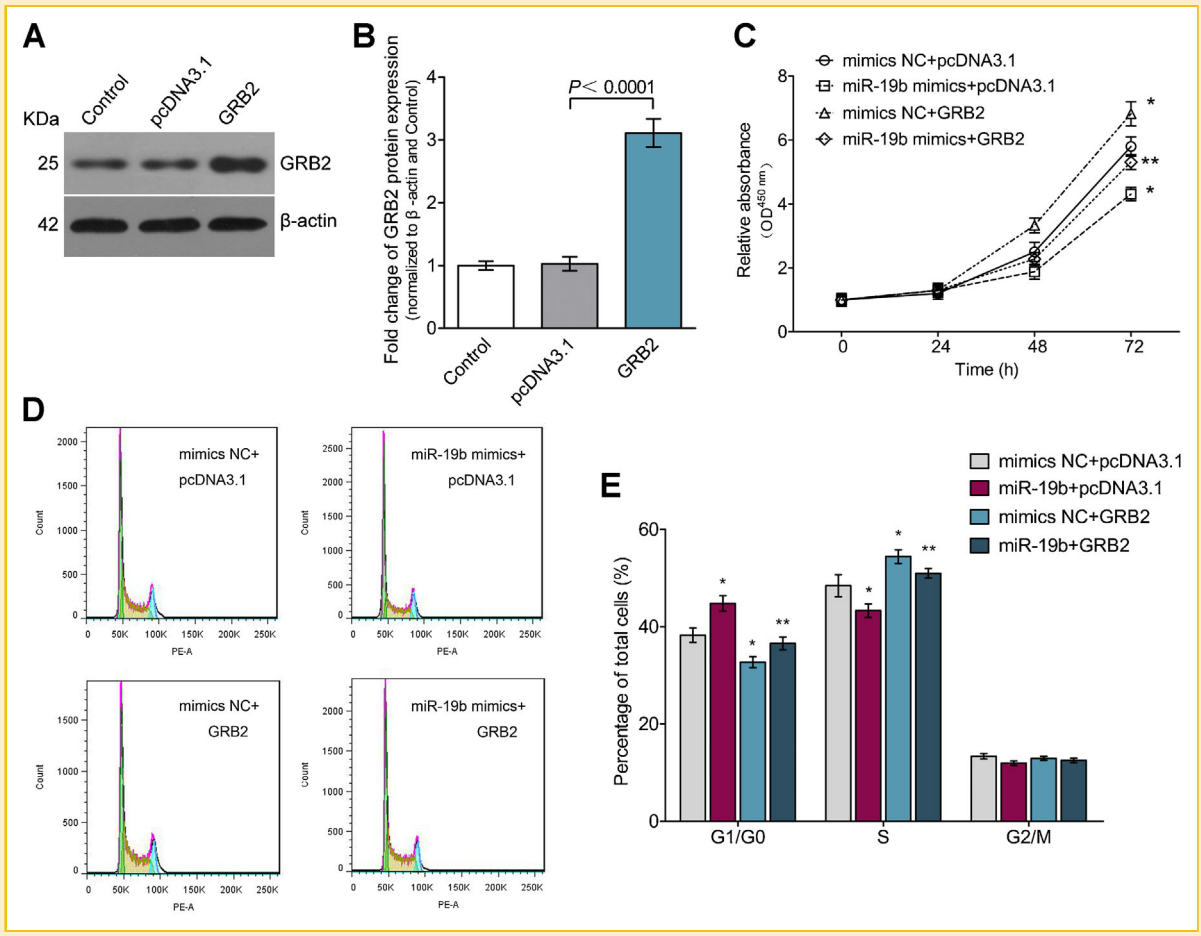

Fig. 5. Forced expression of GRB2 restored the inhibitory effects of miR-19b mimics in proliferation and cell cycle progression of aHSCs. GRB2 expression was analyzed by western blot. (A) GRB2 protein levels were detected by western blot analysis. GRB2 protein levels were up-regulated by transfecting the GRB2 ORF clone. (B) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared with those corresponding to β-actin. The data are presented as the mean ± SEM from 15 replicates in each group. (C) Cell growth of aHSCs was measured by the CCK8 assay. (D) Cell cycle progression was measured using FCM analysis. (E) Percentage of cell cycle distribution. The asterisk indicates a significant difference when miR-19b mimics or GRB2 were compared with miR-SCR; while two asterisks indicate a significant difference when miR-19b mimics + GRB2 were compared with miR-19b mimics or GRB2. (**$P < 0.01$) The figure is representative of 3 experiments with similar results. Values are presented as the mean ± SD.

proliferation as well as decreases in COL1A1 and α-SMA [Venugopal et al., 2010]. Our findings highlighted the importance of miRNA as a potential suppressor in hepatic fibrosis.

GRB2 was a key adapter protein and stimulated cell growth by activating the MAPK/ERK pathway [Scaltriti and Baselga, 2006; McCubrey et al., 2007; Dance et al., 2008], which was crucial in regulating the proportion of hepatocytes in G1/S phase [Talarmin et al., 1999; Rescan et al., 2001]. Likewise, the suppression of GRB2 expression in HepG2 cells reduced fat accumulation, improved glucose metabolism, and ameliorated oxidative stress [Shan et al., 2013]. Additionally, GRB2 action was essential in cardiac fibrosis to regulate fibrosis and cardiomyocyte growth [Zhang et al., 2003]. In lung fibrosis, the knockdown of GRB2 protein levels disrupted c-Met receptor downstream signaling, which resulted in diminished HGF-induced ERK-1/2 phosphorylation and in the recovery of HGF-inhibited expression of MMP-1, NF-kappaB, collagen, and CTGF [Bogatkevich et al., 2007]. Our results obtained from CCK8, cell cycle and western blot assays showed that ectopic miR-19b expression inhibited aHSC proliferation and COL1A1 protein levels and that the over-expression of GRB2 rescued the effect of miR-19b. These results suggested that miR-19b was involved in the proliferation of aHSCs by targeting GRB2. Additionally, miR-19b levels were down-regulated in fibrosis and ECM remodeling of other tissues/organs (pulmonary, cardiac) [Hardie et al., 2009; van Almen et al., 2011], indicating a highly conserved role for miR-19b in fibrogenesis. Several recent studies have identified miR-433 [Luo et al., 2009], miR-200a [Liu et al., 2013], and miR-376c [Iwaki et al., 2013] as suppressors of GRB2. The down-regulation of these miRNAs enhanced GRB2 gene expression and led to cell proliferation, differentiation, and migration, suggesting that GRB2 was tightly regulated by several different miRNAs that include miR-19b. Our findings highlight the importance of the miR-19b/GRB2 axis as a potential pathway in liver fibrogenesis.

In summary, we found that E2-regulated miR-19b was up-regulated in estradiol-treated liver tissues and aHSCs, resulting in inhibition of cell proliferation via the down-regulation of GRB2, which impeded cell proliferation. Moreover, our study highlighted a

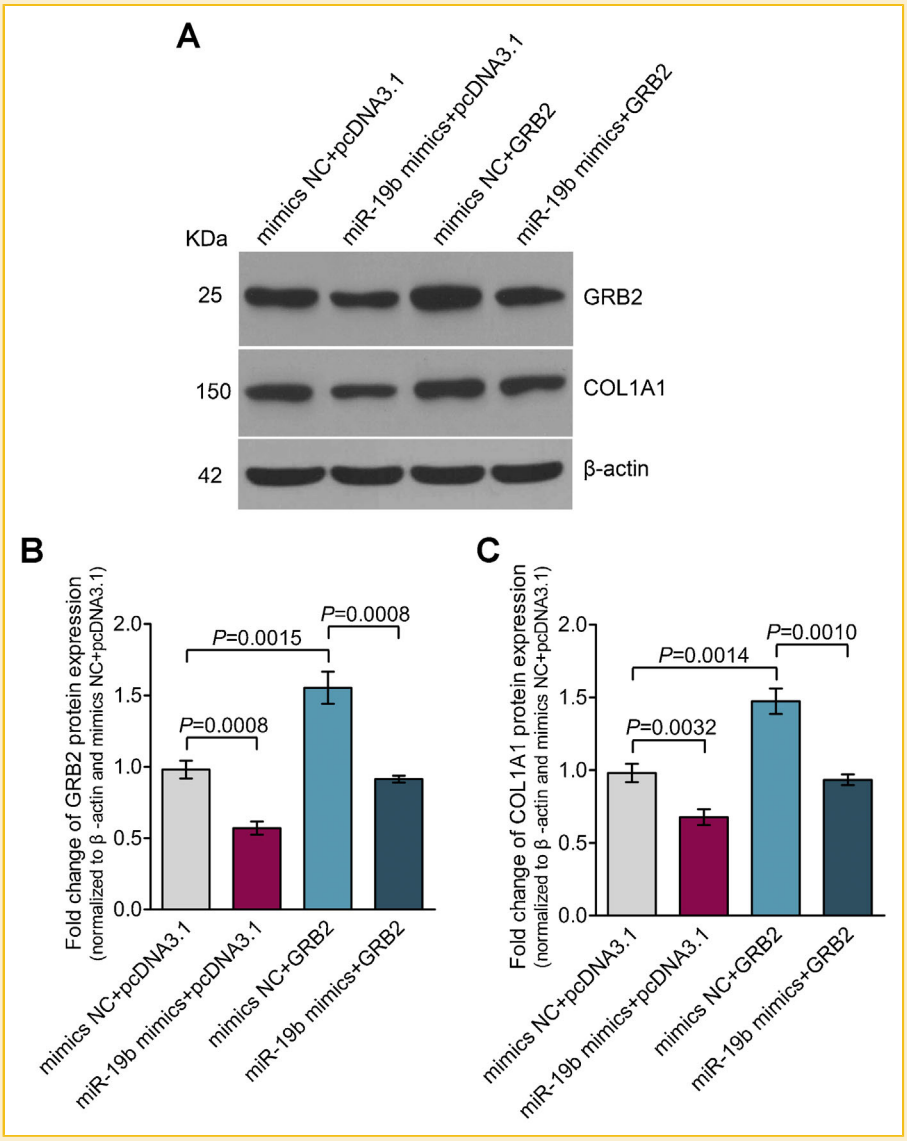

Fig. 6. Forced expression of GRB2 restored the effects of miR-19b mimics in COL1A1. (A) GRB2 and COL1A1 protein levels were detected by western blot analysis. (B) Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to GRB2 were compared with those corresponding to β-actin. Up-regulation of GRB2 reversed the inhibitory effect of miR-19b mimics in levels of GRB2 protein expression. (C) Up-regulation of GRB2 reversed the inhibitory effect of miR-19b mimics in levels of COL1A1 protein expression. The figure is representative of three experiments with similar results. Values are presented as the mean ± SD.

novel miR-19b/GRB2 pathway involved in the estradiol-mediated reduction of HSC proliferation, which might provide a new strategy for miRNA-based anti-fibrotic drug research.

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